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Biological Production of Acetaldehyde from Ethanol Using Non-Growing *Pichia pastoris* Whole Cells

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ABSTRACT

Acetaldehyde has been produced biologically using whole-cell *Pichia pastoris* in a semibatch fermentor. Ethanol and air were fed continuously, and the product, acetaldehyde, was removed by the air stream. Operation of the reactor exceeded 100 h, maintaining high alcohol oxidase activity. Low cell-mass concentration (9.9 g/L) minimized product inhibition. Ethanol concentration in the broth, oxygen concentration in the air, and pH were evaluated for their effects on the fermentation process.

Index Entries: acetaldehyde; *Pichia pastoris;* ethanol; alcohol oxidase; fermentation.

NOMENCLATURE

C_a, C_e	acetaldehyde and ethanol conc. in liquid phase (mol/L).
C_{ai}, C_{ei}	initial acetaldehyde and ethanol conc. (mol/L).
C_a*, C_e*	acetaldehyde and ethanol conc. in gas phase (mol/L).
C_a, in	input acetaldehyde conc. (mol/L).
F	volumetric gas flow rate (L/h).
R_a	rate equation (mol/L/h).

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t	time (h).
V_l, V_g	liquid and vapor phase volume (L).
V_o	reactor total volume (V_g+V_l) (L).
$(dC_a / dt)_{strp}$, $(dC_e / dt)_{strp}$	the stripping rate of acetaldehyde and ethanol (mol/L/h).

INTRODUCTION

Acetaldehyde is an important feedstock in the chemical industry as an intermediate in the manufacture of acetic acid, acid anhydride, butyl alcohol, butyraldehyde, chloral, pyridines, and other derivatives. Acetaldehyde is produced synthetically from the catalytic oxidation of either ethyl alcohol or ethylene. Recent interest has focused on the production of acetaldehyde by biological methods as a natural additive for various foods. Consumer preference for natural products has caused increasing demand for natural flavors. The enzyme alcohol oxidase, in the peroxisome of *Pichia pastoris* yeast could be used to produce a "food-grade" acetaldehyde, which may be more easily marketable to consumers.

The biological process for the production of acetaldehyde is particularly interesting, since acetaldehyde can be removed easily from the fermentor by virtue of its high volatility. A liquid-phase bioreactor operating in semibatch mode is a very simple reaction scheme: only ethanol and air enter, and only acetaldehyde, water, air, and unreacted ethanol leave.

The primary objective of this project has been to quantify the reaction parameters required to conceptually design a commercial reactor. The secondary objective has been to demonstrate enzyme stability in extended operation. A preliminary process design and economic evaluation are presented.

BACKGROUND

Acetaldehyde is generated in metabolic processes and occurs naturally in many fruits and other foods (*1*). Those familiar with fruit flavors recognize that the presence of acetaldehyde is essential for delivering the taste of freshness. Acetaldehyde, a colorless liquid that boils near room temperature, at 21°C, has a suffocating odor that is somewhat fruity and pleasant in dilute concentration. Mixtures of vapor with air are highly flammable and explosive. Acetaldehyde is a highly reactive compound showing the general reactions of aldehydes: it undergoes several condensation, addition, and polymerization reactions (*2*).

A common characteristic of methanol-grown methylotrophic yeasts is the development of unique subcellular compartments, called microbodies, that have a large variety of enzymatic functions. Peroxisomes are microbodies containing alcohol oxidase, the first enzyme in the dissimilatory

pathway for the use of a sole carbon source for growth. Alcohol oxidase operates under a repression/depression metabolic control system. During the exponential growth of methylotrophic yeast on glucose, peroxisomes are difficult to detect, and their physiological function is uncertain. However, when cells are grown with methanol as the carbon source, a number of large peroxisomes are present. If methanol-grown cells are transferred to glucose media, the peroxisomes quickly disappear (*3*).

Alcohol oxidase displays broad specificity and stability. However, activity decreases precipitously with alcohol chain length beyond that of ethanol (*4–6*). Several researchers (*7–11*) have investigated potential uses (a) as enzyme-based electrodes for analytical assay, (b) as an oxygen scavenger, (c) as a food additive, flavor, or fragrance, (d) for sterilization of heat- or radiation-sensitive materials through the release of formaldehyde, and (e) as an ethanol-recovery system.

Ethanol is converted to acetaldehyde in the presence of alcohol oxidase, and the hydrogen peroxide produced is decomposed by catalase into oxygen and water. The enzymatic reactions are:

$$C_2H_5OH + O_2 \xrightarrow{\text{alcohol oxidase}} CH_3CHO + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + 1/2\ O_2$$

The overall reaction is

$$C_2H_5O_2 + 1/2\ O_2 \rightarrow CH_3CHO + H_2O$$

Alcohol oxidase generates harmful hydrogen peroxide, which is highly inhibitory to enzyme activity. However, since the amount of catalase in the peroxisome is far larger than the amount of alcohol oxidase, the H_2O_2 is readily disposed of, and the inhibition of alcohol oxidase is rarely observed (*12–14*). The rate-determining reaction is the oxidation of ethanol by alcohol oxidase.

The significant factors influencing bioreactor performance include temperature, pH, oxygen limitation, and alcohol oxidase deactivation. These variables are discussed as follows:

1. Temperature: At elevated temperatures, enzymatic reaction rates can be accelerated and enzyme contamination reduced (*15*). However, high temperature will deactivate the enyzme, especially for soluble alcohol oxidase, which shows higher temperature sensitivity than does the immobilized form. The optimal temperature for *Pichia pastoris* non-growing whole cells is in the range from 30 to 37°C (*5,10,13,16,17*).
2. pH: In the initial stage of this biological reaction, hydrogen peroxide is produced by the oxidation of ethanol to acetaldehyde, thus pH is expected to decrease slightly because of a

high initial production rate. However, when steady state is reached, the pH should be nearly unchanged.

3. Oxygen limitation: Relatively little is known about the kinetics of alcohol oxidase with respect to its second substrate, oxygen. In the case of *H. polymorpha* and *P. pastoris*, the enzyme has little affinity for oxygen, a property shared by other oxidases that produce hydrogen peroxide (*18*). Oxygen concentration plays an important role, since the enzyme has maximal activity when the aqueous environments are saturated with oxygen. High oxygen is likely to tear down the cell wall and offer a better catalytic reaction area between enzyme and substrate. However, enzyme stability is greatly reduced, probably because of the simultaneous inhibition by acetaldehyde and hydrogen peroxide, which is produced in larger quantities. Thus far, most workers have investigated the production of acetaldehyde by methylotrophic yeast with excess oxygen, and extending alcohol oxidase stability and operation is seldom mentioned.
4. Deactivation: There are several chemicals that will deactivate alcohol oxidase: ethanol, hydrogen peroxide, and acetaldehyde. Since the peroxisome of *P. pastoris* is full of catalase, hydrogen peroxide inhibition might not have a critical effect on this enzyme. Ethanol inhibition appears only slightly in the concentration range from 10 to 60 g/L (*19–21*). However, acetaldehyde has a significant effect on alcohol oxidase deactivation. Duff and Murray (*10*) have shown that 1.0 g/L initial acetaldehyde concentration causes 8% oxidase inhibition and that 4.0 g/L causes 50% inhibition.

Regarding catabolite inactivation, no report for *P. pastoris* has been published. However, when *C. boidinii* and *H. polymorpha* were transferred from the late exponential-growth phase into ethanol-containing media, both catalase and alcohol oxidase activities decreased dramatically. Six hours after the transfer, alcohol oxidase activity was no longer detectable. This inactivation was attributable, at least partially, to the overproduction of acetaldehyde when methanol-grown yeast cultures had been changed to ethanol medium (*10*).

METHODS

Two apparatuses were used to study the enzymatic reaction of converting ethanol to acetaldehyde. Liquid-phase bioreactors were essentially continuous stirred-tank reactors (CSTR) operated in batch mode with respect to cell mass. The first apparatus was a 2-L glass fermentor with a 700-mL working volume, used to explore the general behavior of this enzymatic reaction. The second apparatus was a 5-L New Brunswick

fermentor with a 2-L working volume and better mixing and aeration characteristics than the first reactor. In addition, the 5-L reactor had continuous pH and oxygen monitoring. The fermentors were sterilized prior to the addition of cell mass.

Initially, an isopropanol cold trap was used to collect the highly volatile acetaldehyde. However, the recovery efficiency was not satisfactory. Next, a liquid-nitrogen cold trap reduced the condenser temperature to −75°C. The solution collected during the first 4 h of any experiment was at least 85% acetaldehyde, but the total recovery efficiency for a complete reaction was only about 9% because of continuous air flow through the trap, which removed the volatile acetaldehyde even at these low temperatures.

The fermentor was initially filled with 80% distilled water, 10% whole cell broth, and 10% ethanol, by volume. The substrate, ethanol, was added to the reactor intermittently to maintain ethanol concentration at ±10% of the initial value. The overhead vapor (ethanol, water, and acetaldehyde) was removed continuously by gas flow through the vessel and collected in the nitrogen cold trap. The gas was air, oxygen, or a mixture of both. An oxygen probe monitored the enzyme activity, and pH was monitored and adjusted manually. The temperature was regulated at 30°C and the stirrer speed was controlled at 600 rpm.

This type of liquid-phase bioreactor is not new. Others have examined the removal of alcohol vapor from a fermentor. There is a distinct advantage for acetaldehyde production, since the boiling point of acetaldehyde is 21°C, which is substantially below the boiling points of ethanol and water. This allows the reactor to act as the first separation stage for product purification.

All *P. pastoris* cells used in this study were obtained from Phillips Petroleum Company. Most cells were frozen at −10°C before use. Buffer solution prepared from potassium phosphate, dibasic and monobasic, gave the desired pH range, from 7.0 to 8.0. Cell mass was determined by dry cell weight (DCW). Biomass loss was negligible during all experiments and was between 1 and 3%. Liquid samples of broth and "condensed-off" gases were injected into a gas chromatograph for quantification.

EXPERIMENTAL RESULTS

The operating conditions for all 16 experiments are summarized in Table 1. The first six experiments were performed in the 2-L fermentor. The initial acetaldehyde production rate was defined as the amount of acetaldehyde produced per liter in the first hour. The effect of substrate concentration on the initial acetaldehyde production rate (g acetH/L/h) of *P. pastoris* was studied at a fixed cell concentration (55 g DCW/L). Initial acetaldehyde production increased with ethanol concentration, rising to a maximum near 10% ethanol by volume. The pH was not adjusted initially and remained constant at 5.5. Air flow rate was 2 L/min. Acetaldehyde

TABLE 1
SUMMARY OF EXPERIMENTAL OPERATING CONDITIONS

Expt*	pH	Air Rate (l/min)	O_2 Rate (l/min)	Stirrer (rpm)	DCW (g/l)	Ethanol (g/l)
1	5.5-6.0	2.0	---	600	55	40
2	5.5-6.0	2.0	---	600	55	80
3	5.5-6.0	2.0	---	600	55	160
4	5.5-6.0	2.0-3.5	---	600	55	80
5	5.5-6.0	2.0-4.0	---	600	55	80
6	5.5-6.0	---	2.0-3.5	600	55	80
7	7.0-7.3	2.0	---	200	9.9	80
8	7.1-7.3	2.0-4.0	---	200	9.9	80
9	7.5-7.6	2.0-3.5	---	200	9.9	80
10	7.5-7.6	2.0	---	200	9.9	80
11	7.5-7.6	1.0	1.0	200	9.9	80
12	7.5-7.6	0.5	1.5	200	9.9	80
13	7.5-7.6	1.0	3.0	200	9.9	80
14	7.5-7.6	2.0	---	200	9.9	80
15	7.5-7.6	2.0	---	200	9.9	40
16	7.5-7.6	2.0	---	200	9.9	120

* Expt 1-6 in two liter fermentor, Expt 7-16 in five liter fermentor.

reached a maximum concentration of 12 g/L in 8 h and dropped to near zero after 20 h. Acetaldehyde is well known to have a high inhibitory effect on alcohol oxidase activity (*10*). Experiments 4 and 5 were an attempt to maintain alcohol oxidase activity over a longer period by adjusting the air flow rate to strip acetaldehyde from the reactor and reduce inhibition. If acetaldehyde concentration could be maintained below 1.0% by volume (7.8 g/L), the activity might be extended. However, for these experiments, the initial acetaldehyde production was so high that concentration reached 4.0 g/L in the first hour, and exceeded 1% after 3 h. With this reactor, air flow rate was difficult to adjust, and it was difficult to maintain the acetaldehyde concentration below 1.0% because of the time delay in solution analysis. When compared with Expt. 2, Expts. 4 and 5 showed slightly greater overall yield, but prolonged reaction time (approx 8 h). This was attributed to oxygen-concentration limitation of the reaction rate and caused Expts. 3–5 to have similar production rates. Experiment 6 was operated with the same conditions used in Expts. 4 and 5, except that air was replaced with pure oxygen. Results showed a marked increase in acetaldehyde concentration, to 15.0 g/L (twice that in Experiment 5) within the first hour. The reaction continued for 100 h, with measurable acetaldehyde production. Comparisons among these experiments are shown in Fig. 1. These experiments clearly indicated that improvements were needed in the aeration system for this fermentation.

A 5-L New Brunswick fermentor with a 2-L working volume was used to overcome mixing inefficiencies. Experiments 7–9 were performed to determine the influence of pH in the reaction. The biomass concentration was reduced to 9.9 g/L dried cell mass, 18% of previous experiments. The results are shown in Fig. 2. Experiments 8 and 9, with well-controlled pH,

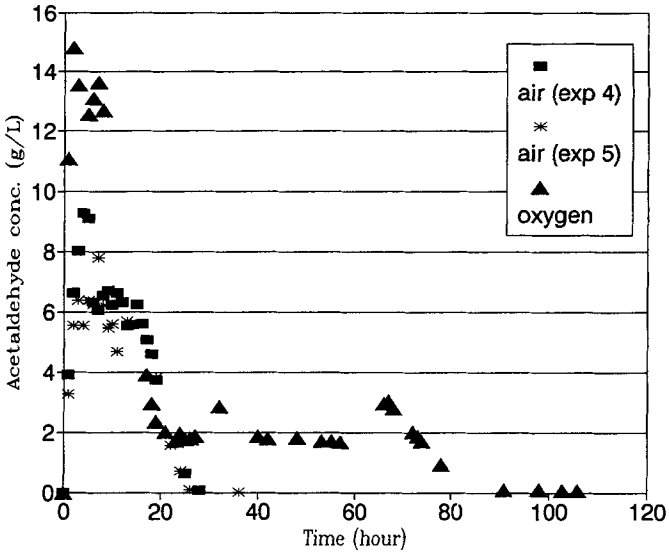

Fig. 1. Acetaldehyde concentration profile in the fermentor with gas flow rate controlled as a function of product in the broth (2.0–3.5 L/min). The biomass concentration was 50% cell broth (DCW = 55 g/L). All other conditions are listed in Table 1.

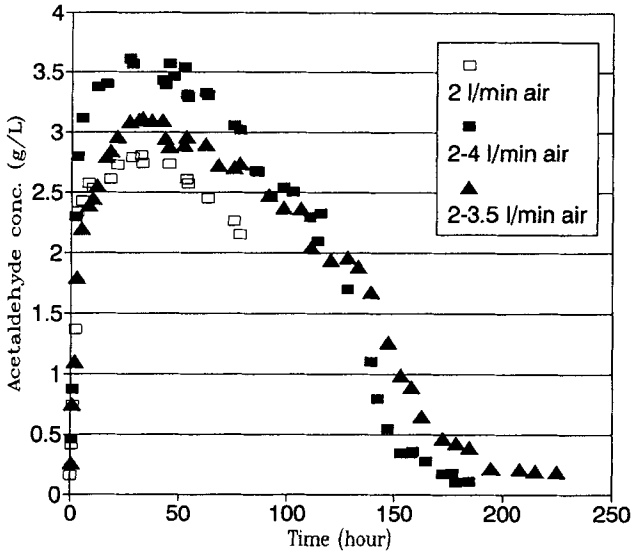

Fig. 2. Acetaldehyde concentration profile in the fermentor for Expts. 7, 8, and 9, with reduced cell-mass concentration (DCW = 9.9 g/L). All other conditions are listed in Table 1.

showed a longer reaction time (over 200 h) than Expts. 4 and 5 (only 36 h), but the initial (first-hour) acetaldehyde production (0.25 g/L in Expt. 8, and 0.47 g/L in Expt. 9) was less than that in Expts. 4 (3.2 g/L) and 5 (3.9 g/L). These differences are the result of the combined effects of cell concentration and fermentor type. Regarding overall acetaldehyde production, which is proportional to the air and acetaldehyde concentration profile

with time, Expts. 8 and 9 were better than Expts. 4–6. Experiment 8 produced 800 g of acetaldehyde during 186 h, and Expt. 9 produced 1050 g of acetaldehyde after 208 h.

A comparison of Expts. 8 and 6 indicates that the fermentation limitation attributable to high oxygen contact can be overcome by reducing the biomass concentration and by using air rather than oxygen, both economically significant factors. The optimum pH appears to be near 7.5, as indicated by the results of Expts. 7–9. Another experiment, at pH 8.0, was abandoned in the fifth hour because of an electrical problem. However, the initial production rate was significantly worse than that of Expt. 9.

To evaluate further the effects of oxygen levels, Expts. 10–13 tested different mixtures of air and oxygen. Pure air (Expt. 10) gave a longer reaction time, but a lower initial acetaldehyde production rate. A high oxygen ratio (Expts. 11 and 12) yielded a higher initial acetaldehyde rate, but a shorter reaction time. Experiment 13 used double total gas flow rate with high O_2 and showed a higher initial rate with a very short reaction time. This is presumably a result of cell damage from higher oxygen contact. The effect of initial ethanol concentration on acetaldehyde reaction was investigated again in Expts. 14–16. The results were consistent with Expts. 1–3. Substrate concentration at 80 g/L (10 vol%) still yielded the best acetaldehyde production.

A mathematical model was developed to describe this fermentation, in order to design a reactor easily. The assumptions used in model development were an isothermal temperature of 30°C, isobaric pressure of 1 atm, completely dried inlet gases, perfect mixing in both liquid and gas phases, homogeneous liquid phase, equilibrium between the gas–vapor mixture and the liquid, the gas–vapor mixture behaving as an ideal gas, the dissolved gas concentration in the liquid remaining constant, and no product holdup within the cells.

The assumption of gas–liquid equilibrium is generally a good approximation in the early stages of this enzymatic reaction. However, as the cell wall begins to break down, this assumption may no longer be correct. Regarding no product holdup within the cells, Duff and Murray (*10*) report about 10% acetaldehyde holdup in whole cells. The model requires material balances for all components. Relations for multicomponent vapor–liquid equilibrium were obtained using Antoine coefficients and the UNIFAC method for the necessary activity coefficients. These procedures result in the following equations:

Material Balance:

$$d(V_l C_a + V_g C_a^*) / dt = F C_{a,in} + R_a V_l - F C_a^* \quad (1)$$

with an initial condition $C_a = C_{ai} = 0$. Expand the LHS, let $V_g = V_o - V_l$, $dV_g/dt = - dV_l/dt$, and define

$$- V_l (dC_a / dt)_{strp} = F C_a^* + V_g (dC_a^* / dt) + (C_a - C_a^*) (dV_l / dt) \quad (2)$$

Then Eq. 1 becomes

$$dC_a / dt = R_a + (dC_a / dt)_{strp} \quad (3)$$

Similarly, for ethanol,

$$-V_l (dC_e / dt)_{strp} = F C_e^* + V_g (dC_e^* / dt) + (C_e - C_e^*) (dV_l / dt) \quad (4)$$

and

$$(dC_e / dt) = R_a + (dC_e / dt)_{strp} \quad (5)$$

with initial (IC) and boundary (BC) conditions

$$\text{IC} \quad C_e = C_{ei} \text{ at } t<0$$
$$\text{BC} \quad C_e = C_{ei} \text{ when } C_e \leq 0.9 \times C_{ei}$$

The boundary condition accounts for intermittent addition of ethanol used during the experiments. Equations 2 and 3 are solved simultaneously knowing the rate expession (R_a), acetaldehyde vapor–liquid equilibrium (C_a and C_a^*), ethanol vapor–liquid equilibrium (C_e and C_e^*), and acetaldehyde and ethanol stripping rates.

The acetaldehyde and ethanol stripping rates can be obtained either from a theoretical derivation or from an experimental correlation. Experimental conditons were used in the previous equations to calculate theoretical stripping rates (simplifying Eqs. 2 and 4).

$$(dC_a / dt)_{strp} = -0.281\, C_a \text{ (mol/L h)} \quad (6)$$

$$(dC_e / dt)_{strp} = -0.023\, C_e \text{ (mol/L h)} \quad (7)$$

In order to evaluate the effectiveness of these equations, experiments without reaction and with known quantities of acetaldehyde and ethanol were performed. A nonlinear least-squares curve fit gave the following expressions:

$$dC_a / dt = -0.293\, C_a \quad (8)$$

$$dC_e / dt = -0.022\, C_e \quad (9)$$

Deviation between the predicted and the measured values for acetaldehyde and ethanol were 4.2 and 4.5%, respectively. The experimental values are used for all subsequent calculations. The constants are a function of flow rate, concentration, temperature, and liquid volume stripped per hour; however, these values should be constant for this scenario.

The plot of maximum acetaldehyde concentration velocity vs initial substrate concentration for Expts. 14–16 shows that the maximum velocity occurs when initial ethanol concentration is about 10%. This indicates that substrate inhibition becomes significant when ethanol concentration is >10%. Product inhibition is also observed. About 10–15% enzyme inhibition at these concentrations is reported by Duff and Murray (*10*). Thus a simultaneous substrate- and product-inhibition kinetic model (*22*) should be developed. The proposed model is:

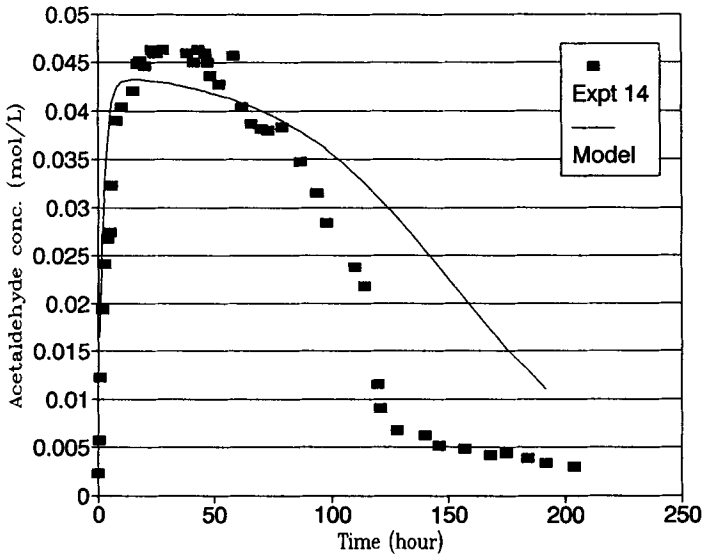

Fig. 3. Comparison of acetaldehyde concentration profile from Expt. 14 with the predictive model. Fermentor conditions are listed in Table 1.

$$R_a = [V_m S / K_s + S + (S^2/K_i)] / \{\exp(-D_i C_a) / [1 - 1/(1 + \exp(-A + Bt)]\} \quad (10)$$

Where the rate constants are $Vm = 0.081$ mol/L, $K_s = 3.06$ mol/L, $K_i = 0.85$ mol/L, $D_i = 5.854$ L/mol, $A = -3.864$, and $B = 0.0275/h$. Equations are solved numerically using a forward finite-difference method. A comparison of the proposed model with experimental data for Expt. 14 is shown in Fig. 3. Departure from experimental data becomes large when operating time is >100 h in consequence of more rapid enzyme deactivation.

This model has been used to consider three cases: a batch reactor, three batch reactors staged for more uniform downstream processing, and a continuous reactor with new cells fed from a seed tank. The assumptions include volumetric air flow rate (L/min) equal to the broth volume, 60% acetaldehyde recovery efficiency, and negligible cell loss during reaction. Operation time for each batch is 120 h (5 d) in case 1 and 168 h (7 d) in case 2. For case 3, the reactor is operated continuously for 300 d. Comparisons are based on a production rate of 473,000 L/yr. The acetaldehyde production rate is FC_a^*. A single fermentor would require a reactor volume of 316,100 L (50 batches/yr). Three batches operating in sequence on an 8-d cycle would require 115,300 L (36 batches/yr each). To operate continuously with 2% new cells fed per withdrawal daily, a reactor volume of 257,000 L would be required.

Process Description

Figure 4 presents a potential process diagram. The *P. pastoris* whole cells are produced in a seed tank (DCW = 120 g/L) and transferred by air pressure to the main fermentor to maintain the 10% whole-cell broth–10%

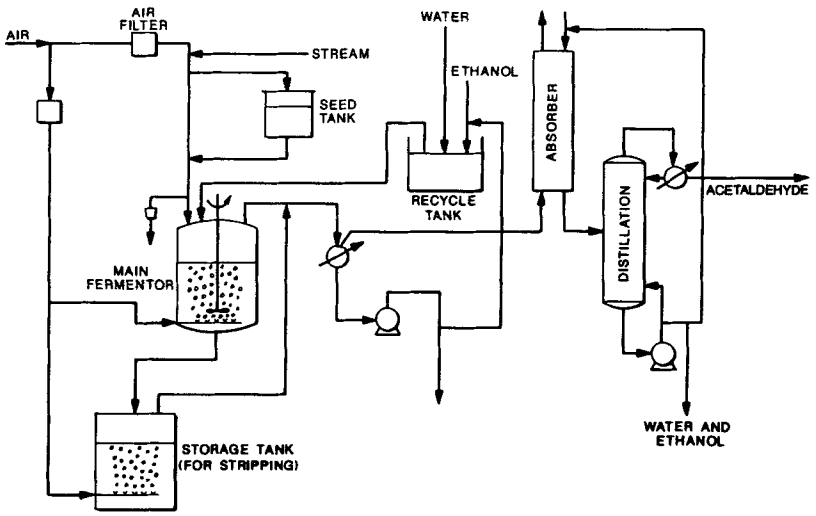

Fig. 4. A proposed process scheme for semibatch production of acetaldehyde from whole-cell *Pichia pastoris* yeast for an annual production rate of 473,000 L/yr.

ethanol–80% water solution. For Process 1, the fermentor operates one batch cycle per week. For the staged-batch operation, each batch runs 8 d plus 1 d off to allow a 3-d lag in start-up.

Process 3 operates continuously 300 d/yr. New cells are fed, beginning in the 61st hour, and a corresponding cell mass is removed from the fermentor. This maintains constant cell activity. Product vapors are sent to a condenser at −10°C, which will condense 6% of the acetaldehyde, 60% of the ethanol, and 80% of the water. Uncondensed acetaldehyde is passed to an additional absorber and distillation column. This unit can be operated at high pressure or with chemical absorbers, such as tris buffer or sulfuric hydroxylamine. We have assumed 60% recovery of the acetaldehyde for these economics, but expect recovery to be significantly higher.

Capital costs for the batch process are listed in Table 2 and the production costs in Table 3. The staged-batch system results in increased capital cost with little increased productivity. The continuous fermentor has high cell costs and lower specific enzyme activity within the fermentor.

The break-even price of acetaldehyde is $4.10/kg, about five times the present acetaldehyde price. With a selling price of $8.80/kg, the pay-back period is about 1 yr, based on an 80% plant financed over 7 yr at an annual interest rate of 15%.

CONCLUSIONS

For the experiments performed in this study, the optimum conditions were pH 7.5 and 10% ethanol concentration. The temperature used, 30°C, is certainly acceptable, but may not be optimum. Substrate, ethanol, and

TABLE 2
CAPITAL COST ESTIMATION FOR BATCH OPERATION
473,000 L/YR ACETALDEHYDE

Item Description	Cost
Fermentor, 320 m^3, 304 SS	$ 300,000
Agitator, 25 hp_1 medium rpm, dual impeller	38,000
Seed tank, 25 m^3, 304 SS	12,000
Mixing tank, 10 m^3, 304 SS	23,000
Storage tank, ethanol, recycle, 100 m^3, 304 SS	30,000
Storage tank, acetaldehyde, 20 m^3, 304 SS	4,000
Sterilizer, 304 SS	30,000
Compressor	20,000
Pump	5,000
Distillation column	65,000
Absorber	120,000
Condenser	16,000
Total	$ 663,000

TABLE 3
PRODUCTION COST ESTIMATION FOR BATCH OPERATION
473,000 L/YR ACETALDEHYDE

Item Description	Cost
Raw Materials	
Pichia whole cells, $ 0.4/kg	$ 422,000
Ethanol, fermentation, $ 0.35/kg	537,000
Utilities and Waste Disposal	180,000
Labor-dependent costs	260,000
Capital-dependent costs	
Maintenance (2% DFC)	13,000
Insurance + taxes (7%)	46,000
Depreciation (15% DFC)	99,000
Total	$ 1,557,000

product (acetaldehyde) inhibitions occur simultaneously in this bioreaction. Acetaldehyde inhibition is severe when the concentration exceeds 4.0 g/L. The alcohol oxidase is sensitive to oxygen concentration, which offers a high production rate but also causes faster enzyme deactivation. Generally we have operated under oxygen limitations in order to prolong enzyme productivity. The proposed model predicts well during the first 120 h of operation; however, there are several limitations. The effect of oxygen concentration on reaction rate has not been addressed; thus, this model can be applied only with air. Also, temperature and agitation speed have been neglected and may be significant design parameters.

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